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The Flavor and Fragrance High Production Volume Consortia

The Cyclohexyl Derivatives Consortium

Test Plan for Alkyl-substituted Cyclohexanol Derivatives

4-*tert*-butylcyclohexanol	**CAS No. 98-52-2**
4-*tert*-butylcyclohexyl acetate	**CAS No. 32210-23-4**

FFHPVC Cyclohexyl Derivatives Consortium Registration Number

Submitted to the EPA under the HPV Challenge Program by:

The Flavor and Fragrance High Production Volume Chemical Consortia

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List of Member Companies

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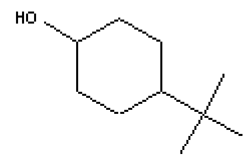
1 IDENTITY OF SUBSTANCES

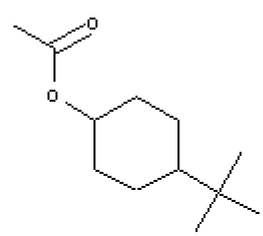


4-*tert*-Butylcyclohexanol

CAS NO. 98-52-2

Synonyms:

Cyclohexanol, 4-(1,1-dimethylethyl)-



4-*tert*-Butylcyclohexyl acetate

CAS NO. 32210-23-4

Synonyms:

Acetic acid, *p-tert*-butylcyclohexyl ester

4-*tert*-Butylhexahydrophenyl acetate

2 CATEGORY ANALYSIS

2.1 INTRODUCTION

In October of 1999, members of the U.S. flavor and fragrance industries as well as other manufacturers that produce source materials used in flavors and fragrances formed consortia of companies in order to participate in the Chemical Right-to-Know Program. Members of these consortia are committed to assuring the human and environmental safety of substances used in flavor and fragrance products. The consortia are organized as the Flavor and Fragrance High Production Volume Consortia (FFHPVC). The cyclohexyl consortium, as a member of FFHPVC, serves as an industry consortium to coordinate testing activities for cyclohexyl substances under the Chemical Right-to-Know Program. Five (5) companies are current members of the Cyclohexyl Consortium. The Cyclohexyl Consortium and its member companies are committed to assembling and reviewing available test data, developing and providing test plans for each of the sponsored chemicals, and where needed, conducting additional testing. The test plan, category analysis and robust summaries presented represent the first phase of the Consortium's commitment to the Chemical Right-to-Know Program.

2.2 BACKGROUND INFORMATION

4-*tert*-Butylcyclohexanol and its corresponding acetate ester, 4-*tert*-butylcyclohexyl acetate are used primarily in soap perfumes. Both substances exist in *cis* and *trans* forms. *trans*-4-*tert*-Butylcyclohexyl acetate exhibits a strong woody aroma while *cis*-4-*tert*-butylcyclohexyl acetate is a more intense woody aroma with a flowery note. The ester has far greater use as a fragrance than does the corresponding alcohol. The alcohol serves as a synthetic precursor of the acetate. Wide variation in the ratio of the *cis* and *trans* isomers does not significantly alter the physical properties. A mixture of *cis* and *trans* alcohol is prepared by hydrogenation of 4-*tert*-butylphenol. The acetate is obtained by acetylation of the *cis* and *trans* mixture of the alcohol.

2.3 STRUCTURAL CLASSIFICATION

This category consists of 2 substances, 4-*tert*-butylcyclohexanol and its corresponding acetate ester, 4-*tert*-butylcyclohexyl acetate. Safety data on the structurally related alkyl-substituted cyclohexanol and cyclohexanone derivatives (*e.g.*, 2-isopropyl-5-methylcyclohexanol, 2-, 3-, and 4-*tert*-butylcyclohexanone and 2-, 3-, and 4-methylcyclohexanone) are also included in this chemical category.

2.4 ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

2.4.1 Hydrolysis of Esters of Cyclohexanol

The unsubstituted monocyclic esters (*e.g*. cyclohexyl acetate) are rapidly hydrolyzed to cyclohexanol and the component aliphatic carboxylic acids by classes of enzymes recognized as carboxylesterases [White *et al.*, 1990; Ford and Moran, 1978; Heymann, 1980], the most important of which are the *beta*-esterases. In mammals, these enzymes occur in most tissues [Anders, 1989; Heymann, 1980] but predominate in the hepatocytes [Heymann, 1980].

Cis- and *trans*-1-methylene-4-isopropenylcyclohexan-2-yl acetate is rapidly hydrolyzed *in vitro* in the presence of rat liver homogenate [Salzer, 1998]. Incubation of the ester resulted in 92% hydrolysis after 15 minutes and 100% after 60 minutes [Salzer, 1998]. The structurally related ethylene glycol and propylene glycol carbonate esters of (-)-2-isopropyl-5-methylcyclohexanol are completely hydrolyzed after incubation for 1 hour with rat liver homogenate [Emberger, 1994]. Sterically hindered esters of cyclohexanol are also readily hydrolyzed in rat liver homogenate. The ester, 3,5,5-trimethyl-[2,3-^{3}H]-cyclohexanyl-[^{14}C]-mandelate (cyclandelate), was completely hydrolyzed to 3,5,5-trimethyl-[2,3-^{3}H]-cyclohexanol and [^{14}C]-mandelic acid within 5 minutes of incubation with microsomal rat hepatocytes. After 20 minutes, 80% of the alcohol disappeared while there was a concomitant linear increase in a *beta*-glucuronidase reactive substance [White *et al.*, 1990]. Presumably, the resulting alcohol was conjugated with glucuronic acid.

Urine collected 18 hours post administration of 350 mg/kg bw of the acetate ester of 2-cyclohexen-1-ol (*i.e.*, cyclohex-1-en-1-yl acetate) to rabbits revealed that 39% of the dose was hydrolyzed and then conjugated with glucuronic acid [Elliott *et al.*, 1959]. Based on this information, it is anticipated that 4-*tert*-butylcyclohexyl acetate is hydrolyzed *in vivo* to yield 4-*tert*-butylcyclohexanol and acetic acid.

Once formed, cyclohexanol or alkyl-substituted cyclohexanols are rapidly absorbed through the gastrointestinal tract and rapidly eliminated from the blood. Peak blood levels are normally reached within 1-2 hours after dosing. Unsubstituted or alkyl-substituted cyclohexanol is rapidly oxidized *in vivo* to the corresponding cyclohexanone derivative by alcohol dehydrogenase. Conversely, the cyclohexanone derivative may be reduced to cyclohexanol by cytosolic carbonyl reductases. Hence, the ketone and alcohol are interconvertible *in vivo*. Conjugation of the alcohol with glucuronic acid and excretion in the bile and urine provides the predominant pathway for metabolic detoxication and elimination of cyclohexanol. Also, since cyclohexanone is readily converted to cyclohexanol and then the glucuronic acid conjugate of cyclohexanol *in vivo*, data on cyclohexanone derivatives are directly relevant to the hazard assessment of cyclohexanol derivatives.

Male Sprague-Dawley rats were exposed to atmospheres of either 400 ppm (240 mg/kg bw) or 1600 ppm (980 mg/kg bw) cyclohexanone for 6 hours. Twenty-four hour post-exposure terminal blood and urine samples show the average plasma levels of cyclohexanone and cyclohexanol for the 400 ppm and 1600 ppm exposures were 26 and 20 micrograms/ml and 122 and 140 micrograms/ml, respectively. The total urinary excretion of cyclohexanol was at least 10 times that of cyclohexanone (16 and 15 micrograms and 143 and 264 micrograms at the 400 and 1600 ppm exposures, respectively) with 13 micrograms and 72 micrograms of conjugated cyclohexanol being excreted within 72 hours at 400 ppm and 1600 ppm, respectively [Topping *et al.*, 1994].

In another study, four rabbits were each given cyclohexanone (No. 1100) in water by gavage. Urine collected at 18 hours after dosing revealed 66% of the 248 mg/kg oral dose was excreted as the glucuronic acid conjugate of cyclohexanol [Elliott *et al.*, 1959]. The authors concluded that cyclohexanone is first reduced to cyclohexanol and then conjugated with glucuronic acid prior to excretion in the urine.

Male beagle dogs were given 284 mg/kg bw of cyclohexanone by intravenous injection daily. Cyclohexanol was detected in the plasma within 30 minutes of injection. The mean distribution and elimination half-lives of cyclohexanone and cyclohexanol are 6.6 and 81 minutes, respectively. The mean steady state volume of distribution for cyclohexanone is 2.6 L/kg and the mean total body clearance for cyclohexanone is 27.4 ml/kg/minutes. [Martis *et al.*, 1980; Koeferl *et al.*, 1981]. When 328 mg/kg bw cyclohexanol was administered by intravenous injection, it showed a plasma half-life of 99 minutes, an apparent distribution volume of 1.2 L/kg and a total body clearance of 8.8 ml/kg/minutes. Based on these data cyclohexanone and cyclohexanol are rapidly cleared from the body [Martis *et al.*, 1980]. Approximately 60% of cyclohexanone administered was recovered in the urine as a glucuronide conjugate of cyclohexanol after 24 hours. The direct renal clearance of unmodified cyclohexanone and cyclohexanol is a minor route of elimination accounting for less than 1% of the administered dose. It is proposed that 74-100% of cyclohexanone is converted to cyclohexanol and further metabolized before elimination. The authors propose that some of the cyclohexanone may be expelled through the lungs [Martis *et al.*, 1980; Koeferl *et al.*, 1981].

Four men and four women volunteers were exposed to an environment containing atmospheric concentration of 101, 207, or 406 mg/cu.m of cyclohexanone for eight (8) hours. Urine collected at 2-hour intervals during exposure and for 72 hours post-exposure show the presence of glucuronic acid conjugates of cyclohexanediol with peak excretion rate at about 16 hours post-exposure. Approximately 60% of the cyclohexanone dose is excreted within the 72-hour period [Mraz *et al.*, 1994].

An adult man ingested 100 ml of liquid adhesive containing 39% cyclohexanone. The cyclohexanone was rapidly absorbed. Plasma and urine levels of cyclohexanone and metabolites were unaffected by gastric lavage (5.5 L saline), two plasma exchanges (2.4 L each) and hemoperfusion when compared to pre-treatment values. Cyclohexanol and cyclohexanone were detected in the plasma for up to 25 hours post ingestion. Cyclohexanone levels were at the lower limit of detection, however, plasma levels of cyclohexanol were high, 220 micrograms/ml 5 hours after ingestion and decreased to 10 micrograms/ml after 20 hours. High levels of cyclohexanol glucuronide were detected in the urine for up to 48 hours. Urinary excretion of the parent ketone was described as minimal. The elimination half-life of cyclohexanone in human plasma was determined at 4.75 hours and the rate of elimination (K_e) 0.145 micrograms/ml/hour. This indicates that the mechanism of elimination in humans involves conversion of the cyclohexanone to cyclohexanol followed by conjugation with glucuronic acid [Sakata *et al.*, 1989].

Other unsubstituted alicyclic ketones (e.g., cyclopentanone) are rapidly absorbed, metabolized, conjugated and excreted mainly in the urine [James and Waring, 1971]. The urine collected from rabbits orally administered 193 mg/kg bw cyclopentanone was taken and treated with *beta*-glucuronidase. The resulting analysis revealed that the major urinary component was a glucuronic acid conjugate of cyclopentanol [James and Waring, 1971].

The size, position, number, or stereochemistry of alkyl substituents on the cyclohexyl ring exerts no significant effect on the rate of absorption, metabolism and excretion of alkyl-substituted cyclohexanol or cyclohexanone derivatives. Alkyl-substituted cyclohexanols are rapidly absorbed, conjugated with glucuronic acid, and excreted mainly in the urine. Alkyl-substituted cyclohexanones are also rapidly absorbed, reduced to the corresponding cyclohexanol derivatives that are then conjugated with glucuronic acid and also excreted mainly in the urine.

The urine of groups (6 to 10) of doe albino rabbits was pooled 24 hours after each animal received a single oral dose of 652 mg/kg bw of (±)-2-*tert*-butylcyclohexanone, 652 mg/kg bw of (±)-3-*tert*-butylcyclohexanone, or 562 mg/kg bw of 4-*tert*-butylcyclohexanone [Cheo *et al.*, 1967]. The mean % of the dose excreted as the glucuronic acid is 76.5, 90, or 80% respectively.

Rabbits given oral doses of 1750 mg/kg bw of methylcyclohexanol (mixture of isomers) or 560 mg/kg bw of methylcyclohexanone (mixture of isomers) predominantly excrete the glucuronic acid of methylcyclohexanol within the first 24 hours [Treon *et al.*, 1943a]. Rabbits were exposed to atmospheres containing 2.3 (503 ppm), 1.06 (232 ppm), or 0.56 mg/L (121 ppm) of methylcyclohexanol (mixture of isomers), 6 hours daily, 5 days per week for 10 weeks. Mean daily urinary output of glucuronic acid conjugates during exposure is proportional to dose. Rabbits exposed to atmospheres containing 2.31 (514 ppm) or 0.816 mg/L (132 ppm) of methylcyclohexanone (mixture of isomers), 6 hours daily, 5 days per week for 10 weeks exhibit mean daily urinary output of glucuronic acid proportional to dose [Treon *et al.*, 1943b].

Rats received 500 mg/kg bw (128 microcurie/mg) of 3-^{3}H-2-isopropyl-5-methylcyclohexanol and urine and feces were collected 24 and 48 hours after dosing. The total excretion of 3-^{3}H-2-isopropyl-5-methylcyclohexanol by intact and bile duct-cannulated rats was greater than 70% of the dose at 48 hours. The glucuronic acid conjugate of 2-isopropyl-5-methylcyclohexanol and other minor oxidized metabolites are present in urine and fecal extracts. The glucuronic acid conjugate is also the main metabolite in the bile, while the glucuronic acid conjugate and minor metabolites (less than 5%) formed by side-chain oxidation are excreted in the urine [Yamaguchi *et al.*, 1994].

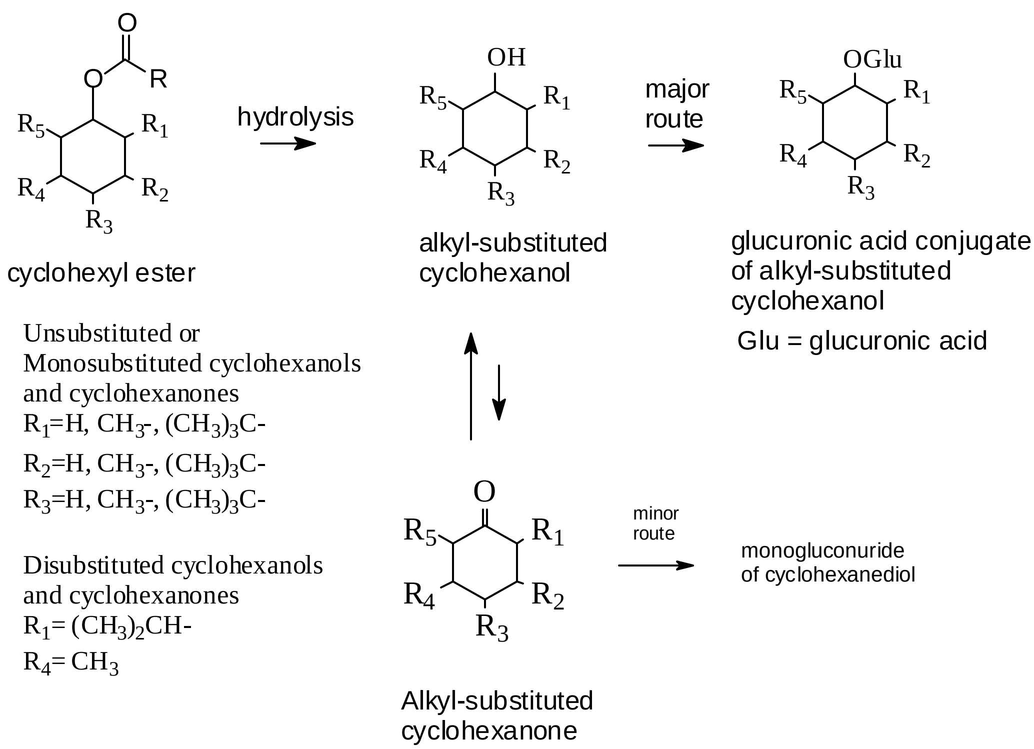
In summary, the esters of cyclohexanol are readily hydrolyzed. In the principal excretion pathway, the cyclohexanols are conjugated with glucuronic acid and excreted primarily in the urine. Also, since alkyl-substituted cyclohexanols are interconvertible with their corresponding ketones *in vivo*, data on alkyl-substituted cyclohexanones are relevant to the evaluation of the 4-*tert*-butylcyclohexanol and its corresponding acetate ester, 4-*tert*-butylcyclohexyl acetate as well.

2.4.2 Metabolism

As indicated above, 4-*tert*-butylcyclohexyl acetate will undergo hydrolysis to yield 4-*tert*-butylcyclohexanol. Subsequently 4-*tert*-butylcyclohexanol is conjugated with glucuronic acid to yielded the corresponding glucuronide that is excreted mainly in the urine. This metabolic pathway can be derived from studies with cyclohexanone derivatives. The major metabolic pathway involves reduction of the cyclohexanones to yield the corresponding cyclohexanols that are subsequently excreted primarily as the glucuronic acid conjugates [Lington and Bevan, 1994; Topping *et al*., 1994; Cheo *et al.*, 1967; Elliot *et al*., 1965; Yamaguchi *et al.*, 1994]. To a very minor extent, alicyclic ketones and secondary alcohols containing an alkyl side-chain undergo oxidation of the side-chain to form polar poly-oxygenated metabolites that are also excreted as the glucuronide or sulfate conjugates mainly in the urine.

Although it has been anticipated that lipophilic alcohols or ketones with sterically hindered functional groups would undergo more extensive oxidation of alkyl ring substituents [Nelson *et al.*, 1992], studies with 2-, 3-, or 4-methylcyclohexanol, 2-isopropyl-5-methylcyclohexanol, 3,5,5-trimethylcyclohexanol, and even 2-, 3-, or 4-*tert*-butyl-substituted cyclohexanol or cyclohexanones reveal that conjugation of the cyclohexanol moiety by glucuronic acid is the predominant excretion pathway regardless of the size or position of the ring substituent. In general, the metabolic fate of alkyl-substituted cyclohexanol and cyclohexanone derivatives is similar to that of the un-substituted homologues (see Figure 1) [Lington and Bevan, 1994; Topping *et al*, 1994].

FIGURE 1. METABOLIC FATE OF CYCLOHEXYL DERIVATIVES IN ANIMALS



In rats and rabbits, 66% of a 186 mg/kg bw dose of cyclohexanone or 47% of a 193 mg/kg bw dose of cyclopentanone *via* gavage is reduced to the corresponding secondary alcohol and excreted in the urine as the glucuronic acid conjugate [James and Waring, 1971]. Also, detected are trace amounts of mercapturic acid conjugate of the 2-hydroxycyclohexyl derivative [James and Waring, 1971]. Eighteen (18)-hour urine samples from rabbits administered 1500 mg of cyclohexanone by gavage contain 65% cyclohexanol and a minor amount (6%) of *trans*-cyclohexane-1,2-diol as monoglucuronide conjugates [Elliott *et al.*, 1959]. Presumably, the diol forms by hydroxylation at the *alpha*-position of cyclohexanone followed by reduction of ketone function. The corresponding cyclohexanol derivative is the major urinary metabolite obtained from rabbits fed 460 mg/kg bw cyclohexane, 260 mg/kg bw cyclohexanol, or 350 mg/kg bw cyclohex-1-en-1-yl acetate [Elliott *et al.*, 1959].

The urine of rabbits given an oral dose of 1200 mg/kg bw of cyclohexanol, shows a significant increase in glucuronic acid conjugates and decrease in inorganic sulfate compared to pre-dose levels [Treon *et al.*, 1943a]. The glucuronic acid conjugate of cyclohexanol is also obtained as the major urinary metabolite in rabbits given 890 mg/kg bw of cyclohexanone [Treon *et al.*, 1943a]. The glucuronic acid conjugate of cyclohexanol (1.55 mg/L) and small amounts of cyclohexanone (0.23 mg/L) were found in the urine of workers occupationally exposed to a mixture of atmospheric hexanes including 456 mg/cu.m of cyclohexane [Governa *et al.*, 1987; Perbellini *et al.*, 1980]. The authors concluded that the cyclohexane is transformed to cyclohexanol that subsequently forms glucuronic acid and sulfate conjugates.

Rats and rabbits were given oral doses of 200 - 3200 mg/kg bw of 2-, 3-, or 4-methylcyclohexanone. The glucuronic acid and sulfate conjugates of the corresponding secondary alcohols were the predominant urinary metabolites [Treon *et al.*, 1943a; Elliott *et al.*, 1959; Tao and Elliott, 1962].

Although the glucuronic acid conjugation of the alcohol is the predominant excretion pathway, oxidation of the alkyl substituents to yield poly-oxygenated metabolites has been reported as a minor pathway in animals. The number of possible polyoxygenated metabolites increases with an increase in the types of alkyl ring substituents (*e.g.*, methyl and isopropyl substituents) [Nelson *et al.*, 1992; Yamaguchi *et al.*, 1994; Madyastha and Srivatsan, 1988; Asakawa *et al.*, 1986].

The glucuronic acid conjugate of 2-, 3-, or 4-*tert*-butylcyclohexanol is the major urinary metabolite obtained 24 hours after rabbits were given 652 mg/kg bw of (±)-2-*tert*-butylcyclohexanone, 652 mg/kg bw of (±)-3-*tert*-butylcyclohexanone, or 562 mg/kg bw of 4-*tert*-butylcyclohexanone, respectively [Cheo *et al.*, 1967]. The mean percent of dose excreted is 76.5, 90, or 80% for 2-, 3-, or 4-*tert*-butylcyclohexanone, respectively. The ratio of *cis*- to *trans*-*tert*-butylcyclohexanol present in the urine of animals given 2-(71:29), 3-(74:26), or 4(26:74)-*tert*-butylcyclohexanone provides evidence that carbonyl reductase catalyzed reduction of the ketone function with NADH is influenced by steric effects of the *tert*-butyl substituent. The authors suggest that NADH uses a perpendicular approach to the carbonyl function in 2- and 3-*tert*-butylcyclohexanone. The 4-*tert*-butyl substituent, being more removed from the reaction site, exerts only a minor impact on stereochemistry of the reduction of the ketone to the alcohol. In contrast, a "face to face" approach is used during the reduction of the corresponding smaller alkyl substituents (*e.g.*, methyl-substituted cyclohexanones) by NADH. In these cases, the *trans* isomer is favored [Elliott *et al.*, 1965].

The presence of multiple alkyl substituents at different positions on the cyclohexyl ring does not significantly alter the principal pathway of metabolism and excretion. 2-Isopropyl-5-methylcyclohexanol is mainly conjugated with glucuronic acid. At higher dose levels, *omega*-oxidation of the side chain substituents occurs to yield various polyols and hydroxyacids of 2-isopropyl-5-methylcyclohexanol [Yamaguchi *et al.*, 1994; Madyastha and Srivatsan, 1988]. The unchanged alcohol and minor metabolites formed by side chain oxidation are eliminated in the urine and feces either unchanged or conjugated with glucuronic acid [Yamaguchi *et al.*, 1994]. The corresponding ketone is primarily reduced to the corresponding secondary alcohol that is then eliminated as noted above [Williams, 1940].

The metabolic fate of 2-isopropyl-5-methylcyclohexanol and 2-isopropyl-5-methylcyclohexanone has been studied in humans and other animals. Seventy-nine percent (79%) of a 1000 mg oral dose [Quick, 1928] or 78% of a 10-20 mg oral dose [Atzl *et al.*, 1972] of 2-isopropyl-5-methylcyclohexanol administered to volunteers is eliminated as the glucuronic acid conjugate. For eight days, 750 mg of the *l* stereoisomer of 2-isopropyl-5-methylcyclohexanol was orally administered to three human volunteers followed by oral or intravenous administration of 200 mg [6-^{13}C]-glucuronolactone or [6-

^{13}C]-sodium glucuronate. Up to 84% of the administered dose of labeled 2-isopropyl-5-methylcyclohexanol is excreted as the glucuronic acid conjugate in the urine after 48 hours [Eisenberg *et al*., 1955]. In two separate studies involving a total of 19 male and female volunteers, the glucuronic acid conjugate of 2-isopropyl-5-methylcyclohexanol is detected in the urine following oral administration of a 180 mg dose of an essential oil (peppermint oil) containing greater than 80% of 2-isopropyl-5-methylcyclohexanol, its stereoisomers, and the corresponding ketone [Kaffenberger and Doyle, 1990]. A 4500 mg/kg bw oral dose of 2-isopropyl-5-methylcyclohexanol administered to rabbits is conjugated with glucuronic acid and eliminated in the urine [Deichmann and Thomas, 1943; Williams, 1939; Quick, 1924].

In rats, the vast majority of orally administered 2-isopropyl-5-methylcyclohexanol is eliminated in either the urine or feces as the glucuronic acid conjugate or, to a lesser extent, as various oxidation products of the alcohol [Yamaguchi *et al*., 1994; Madyastha and Srivatsan, 1988]. Non-cannulated and bile duct-cannulated male Fischer 344 rats (5/sex) were administered a single dose of 500 mg [3-^{3}H]-*l*-2-isopropyl-5-methylcyclohexanol/kg bw. Urine and feces were collected over the next 24 and 48 hours in non-cannulated rats. In the bile duct-cannulated rats, three bile samples were collected in two-hour intervals for the first six hours and a final sample was collected after 24 hours. Urine was collected at 24 hours.

In the non-cannulated rats, total recovery of the labeled substance in the urine or feces is 71.7% with the majority of the dose (45.4%) being recovered within the first 24 hours. In the urine, 37.8% percent of the radioactivity is excreted with equal amounts for the first and second 24 hours. In the feces, 33.9% of the radioactivity is recovered with the majority in the first 24 hours (26.6%) [Yamaguchi *et al.*, 1994]. In the bile duct-cannulated rats, total recovery of the labeled substance in the urine or bile is 74.2% with the majority being recovered in the bile (66.9%). The bile metabolites are mainly the glucuronic acid conjugate of 2-isopropyl-5-methylcyclohexanol along with a variety of oxidation products in which the alkyl substituents (isopropyl or methyl substituents) of 2-isopropyl-5-methylcyclohexanol are oxidized [Yamaguchi *et al.*, 1994].

The biliary route of metabolism of 2-isopropyl-5-methylcyclohexanol appears to be more important in rodents and dogs than in humans and rabbits. *l*-2-Isopropyl-5-methylcyclohexanone given to rabbits (1000 mg/kg bw) [Williams, 1938, 1940] is stereoselectively reduced to *d* stereoisomer of 2-isopropyl-5-methylcyclohexanol [Williams, 1940].

Urine samples collected over the course of four (4) days from rabbits given 1000 mg/kg bw of isophorone (3,5,5-trimethyl-2-cyclohexen-1-one) *via* gavage showed several metabolites: the three major conjugated metabolites include 3,5,5-trimethyl-2-cyclohexen-1-ol (isophorol), formed by reduction of the ketone group and then conjugation with glucuronic acid, *cis*- and *trans*-3,5,5-trimethylcyclohexanol formed by hydrogenation of the endocyclic double bond, reduction of the ketone, and conjugation with glucuronic acid, and 5,5-dimethyl-1-cyclohexene-3-one-1-carboxylic acid formed by methyl group oxidation at an exocyclic allylic position [Truhaut *et al*., 1970; Dutertre-Catella *et al*, 1978].

The data clearly demonstrate that unsubstituted or alkyl-substituted cyclohexanones are readily reduced to the corresponding cyclohexanol derivatives in a variety of animal species over a wide range of dose levels. The cyclohexanol derivatives are then conjugated with glucuronic acid and excreted mainly in the urine.

3 TEST PLAN

3.1 CHEMICAL AND PHYSICAL PROPERTIES

3.1.1 Melting Point

The melting point of 4-*tert*-butylcyclohexanol has been reported to be 56.6-58.6 ºC [Krestinina *et al*., 1984], 56-58 °C [General Aniline and Film Corp., 1965] and 55-70 ºC [Fragrance Materials Association (FMA), unpublished report], and has been calculated to be 4.34 °C [MPBPVPWIN EPI Suite, 2000].

The melting point of 4-*tert*-butylcyclohexyl acetate has been reported to be less than or equal to -50 ºC [Degussa AG, 2003b] and has been calculated to be 10.93 C [MPBPVPWIN EPI Suite, 2000].

The melting point of the isomeric alkyl-substituted cyclohexanol, 2-isopropyl-5-methylcyclohexanol was reported to be 41-43 ºC [Merck Index, 1997], 30 ºC (synthetic menthol) and 41 ºC (natural menthol) [Fragrance Materials Association (FMA), unpublished report].

Based on the above data, the melting points of 4-*tert*-butylcyclohexanol and 4-*tert*-butylcyclohexyl acetate are 56.6-58.6 ºC and less than or equal to -50 ºC, respectively.

3.1.2 Boiling Point

The boiling point of 4-*tert*-butylcyclohexanol has been reported to be 223-228 ºC at 1013 hPa [Degussa AG, 2003a, 1998a] and 110 ºC at 15 mm Hg [Fragrance Materials Association (FMA), unpublished report], and has been calculated to be 216.91 ºC [MPBPVPWIN EPI Suite, 2000].

The boiling point of 4-*tert*-butylcyclohexyl acetate has been reported to be approximately 241 ºC at 1013 hPa [Degussa AG, 2003b, 1998b] and 260 ºC [Fragrance Materials

Association (FMA), unpublished report], and has been calculated to be 232.55 ºC [MPBPVPWIN EPI Suite, 2000].

The boiling point of isomeric cyclohexanol 2-isopropyl-5-methylcyclohexanol was reported to be 212 ºC [Merck Index, 1997] and 216 ºC [Fragrance Materials Association (FMA), unpublished report]. Based on the measured boiling points values from a number of sources, the boiling points of 4-*tert*-butylcyclohexanol and 4-*tert*-butylcyclohexyl acetate are 224-228 ºC and 241 ºC, respectively.

3.1.3 Vapor Pressure

The vapor pressure of 4-*tert*-butylcyclohexanol has been reported to be less than 0.1 kPa or less than 0.75 mm Hg at 20 ºC [Degussa AG, 2003a]. The calculated vapor pressure has been estimated to be 0.005 mm Hg at 20 ºC [Fragrance Materials Association (FMA), unpublished report]. The calculated vapor pressure for 4-*tert*-butylcyclohexanol according to the MPBPVPWIN program is 0.0263 mm Hg at 25 °C.

The vapor pressure of 4-*tert*-butylcyclohexyl acetate has been reported to be 0.01 hPa or less than 0.075 mm at 20 ºC [Degussa AG, 2003b] and has been calculated to be approximately 0.067 kPa or less than 0.050 mm Hg at 20 ºC [Huels AG, 1985] and 0.03 mm Hg at 20 ºC [Fragrance Materials Association (FMA), unpublished report].

The calculated vapor pressure for 4-*tert*-butylcyclohexyl acetate according to the MPBPVPWIN program is 0.002 kPa or 0.0159 mm Hg at 25 °C.

The vapor pressure of isomeric cyclohexanol, 2-isopropyl-5-methylcyclohexanol, was reported to be 0.02 mm Hg at 20 ºC [Fragrance Materials Association (FMA), unpublished report]. Based on the experimental and calculated data, the vapor pressure of 4-*tert*-butylcyclohexanol is less than 0.1 kPa or less than 0.75 mm Hg at 20 ºC and vapor pressure of 4-*tert*-butylcyclohexyl acetate is 0.01 kPa or less than 0.075 mm Hg at 20 ºC

3.1.4 n-Octanol/Water Partition Coefficients

Log K_{OW} for 4-*tert*-butylcyclohexanol calculated by different models, resulted in values of 3.42 [KOWWIN EPI Suite, 2000]. The calculated value exhibits good agreement with the measured log K_{OW} value 3.23 [Degussa AG, 1981]. The measured value for 4-*tert*-butylcyclohexanol is in good agreement with the calculated log K_{OW} value of 3.38 for the isomeric cyclohexanol derivative, 2-isopropyl-5-methylcyclohexanol [KOWWIN EPI Suite, 2000].

For 4-*tert*-butylcyclohexyl acetate, the calculated log K_{OW} value of 4.42 [KOWWIN EPI Suite, 2000] is slightly less than the measured value of 4.8 [Givaudan-Roure, 1996].

Based on these data the log K_{OW} values for 4-*tert*-butylcyclohexanol and 4-*tert*-butylcyclohexyl acetate are 3.23 and 4.8, respectively.

3.1.5 Water Solubility

Reported experimental values for water solubilities for 4-*tert*-butylcyclohexanol and 4-*tert*-butylcyclohexyl acetate are less than 100 [Degussa AG, 2003a] and approximately 90 mg/L [Degussa AG, 2003b] at 20 ºC, respectively while calculated values are determined to be 528.9 and 2.552 mg/L, respectively, at 25 ^{o}C [WSKOWIN EPI Suite, 2000]. Based primarily on reported experimental values, the water solubility of the alcohol is concluded to be less than 100 mg/L at 20 ºC and water solubility of the acetate is concluded to be 90 mg/L at 20 ºC.

3.1.6 New Testing Required

No further testing is required.

3.2 ENVIRONMENTAL FATE AND PATHWAYS

3.2.1 Photodegradation

The calculated half-life values for 4-*tert*-butylcyclohexanol and 4-*tert*-butylcyclohexyl acetate have been reported to be 6.361 and 8.850 hours, respectively [AOPWIN EPI Suite, 2000]. The calculations are based on measured rate constants for radical reactions of OH, O_3 and NO_3 with organic substrates [AOPWIN EPI Suite, 2000]. The short half-life for the alcohol is consistent with the presence of reactive alcoholic OH function. Therefore, the half-life can be considered reliable.

3.2.2 Stability in Water

The calculated hydrolysis half-life for 4-*tert*-butylcyclohexyl acetate is 266 days at pH 8 and 7.2 years at pH 7 [HYDROWIN EPI Suite, 2000]. Other cyclohexanol esters are readily hydrolyzed *in vivo* (see Hydrolysis Section 2.4.1.)

3.2.3 Biodegradation

In a study adhering to OECD Guidelines, 4-*tert*-butylcyclohexanol was readily biodegradable (90% in 19 days) when tested using predominantly domestic sewage [Degussa AG, 1983].

4-*tert*-Butylcyclohexyl acetate was reported to be readily biodegradable (*i.e.*, >60% biodegradation within 10-day window) when tested with domestic activated sludge in the ISO BOD test for insoluble substances (68% in 28 days) [Degussa AG, 1995] and an aerobic evolution test (75% in 28 days) [Degussa AG, 1997b].

In the Manometric Respirometric test, 4-*tert*-butylcyclohexyl acetate was not readily biodegradable (54% after 28 days) [Rudio, 1996a], and was not inherently but partially biodegradable (24% after 28 days) when determined by the Respirometric Method (modified MITI Test II) [Rudio, 1996b].

Given the database of information, 4-*tert*-butylcyclohexanol and 4-*tert*-butylcyclohexyl acetate are readily biodegradable.

3.2.4 Fugacity

Transport and distribution in the environment were modeled using Level III Fugacity-based Environmental Equilibrium Partitioning Model [Mackay, 1991, 1996a, 1996b] through the EPA EPI Suite 2000 program. The input parameters used were molecular weight, melting point and boiling point.

The model predicts that 4-*tert*-butylcyclohexanol is distributed mainly to the soil (59.7%), but also is distributed to water (37.9%) and, to a small extent, air (1.92%) and sediment (0.468%). In addition, the model predicts that 4-*tert*-butylcyclohexyl acetate is distributed mainly to the soil (71.3%), but also is distributed to water (14.9%) and sediment (12.1%) and, to a lesser extent, air (1.66%).

In these environmental compartments, released 4-*tert*-butylcyclohexanol exhibits a potential to be oxidized to the corresponding ketone while 4-*tert*-butylcyclohexyl acetate is appreciably hydrolyzed to the alcohol that is then oxidized to the ketone. Because of their use in cosmetics, soaps and detergents, the majority of 4-*tert*-butylcyclohexanol will enter the environment primarily *via* a sewage treatment plant and will be rapidly and extensively biodegraded. Therefore, low levels of the alcohol and ester will reach the environment.

3.2.5 New Testing Required

No further testing is required.

3.3 ECOTOXICITY

3.3.1 Acute Toxicity to Fish

Experimental and calculated acute toxicity data for fish were available for 4-*tert*-butylcyclohexanol and 4-*tert*-butylcyclohexyl acetate. In fresh water fish, the 48-hour static LC50 for 4-*tert*-butylcyclohexanol was reported to be 17 mg/L [Degussa AG, 1987]. Similarly, for 4-*tert*-butylcyclohexyl acetate, the 48-hour static LC50 was reported to be 14 mg/L [Degussa AG, 1985a] and a 96-hour semi-static LC50 was reported to be 8.6 mg/L [Degussa AG, 1997c].

For 4-*tert*-butylcyclohexanol, the calculated 96-hour LC50 was reported to be 8.085 mg/L (neutral organics) and the 14-day LC50 was calculated to be 17.805 mg/L [ECOSAR EPI Suite, 2000]. For 4-*tert*-butylcyclohexyl acetate, the calculated 96-hour LC50 was reported to be 0.954 mg/L (esters) [ECOSAR EPI Suite, 2000].

Given the consistency of measured and calculated data, it will not be necessary to perform additional acute fish toxicity tests. The 48-hour static LC50 for 4-*tert*-butylcyclohexanol and 4-*tert*-butylcyclohexyl acetate are 14-17 mg/L. The 96-hour semi-static LC50 is expected to be approximately 10 mg/L for 4-*tert*-butylcyclohexyl acetate.

3.3.2 Acute Toxicity to Invertebrates

Measured and calculated aquatic invertebrate LC50 values were available for 4-*tert*-butylcyclohexanol and 4-*tert*-butylcyclohexyl acetate. In *Daphnia magna,* the 48-hour EC50 values were determined to be 46 and 23.4 mg/L for 4-*tert*-butylcyclohexanol and 4-*tert*-butylcyclohexyl acetate, respectively [Degussa AG, 1994b, 1997a]. In two other studies, the 24-hour EC50 values for 4-*tert*-butylcyclohexyl acetate was determined to be 7.0 mg/L [Degussa AG, 1985b] and 19 mg/L [Degussa AG, 1985c].

In *Daphnia magna,* the calculated 48-hour LC50 values for 4-*tert*-butylcyclohexanol and 4-*tert*-butylcyclohexyl acetate were reported to be 9.431 mg/L and 0.446 mg/L, respectively [ECOSAR EPI Suite, 2000]. In mysid shrimp, a 96-hour LC50 value of 0.969 mg/L was calculated for 4-tert-butylcyclohexanol [ECOSAR EPI Suite, 2000].

3.3.3 Acute Toxicity to Aquatic Plants

Experimental and calculated acute toxicity data for aquatic plants were available for 4-*tert*-butylcyclohexanol and 4-*tert*-butylcyclohexyl acetate. 4-*tert*-Butylcyclohexanol and 4-*tert*-butylcyclohexyl acetate were tested in *Scenedesmus subspicatus* (algae) and, based on growth rates, the 72-hour EC50 values of 45 and 17 mg/L, respectively, were determined [Degussa AG, 1992, 1994a].

Calculated 96-hour EC50 values of 6.329 mg/L for 4-*tert*-butylcyclohexanol and 0.084 mg/L for 4-*tert*-butylcyclohexyl acetate are at least an order of magnitude less than the experimentally determined values for green algae. This reflects the conservative nature of the model predictions [ECOSAR EPI Suite, 2000].

3.3.4 New Testing Required

No further testing is required.

3.4 HUMAN HEALTH

3.4.1 Acute Toxicity

Numerous oral, dermal, and intraperitoneal LD50 values for 4-*tert*-butylcyclohexanol and 4-*tert*-butylcyclohexyl acetate have been reported in rats, mice, and rabbits and have demonstrated the overall low acute toxic potential of chemicals in this category. Oral and dermal LD50s tended to exceed 4,000 mg/kg bw, whereas intraperitoneal LD50s in mice were much lower (less than 400 mg/kg bw).

For 4-*tert*-butylcyclohexanol, the rat oral LD50 was reported to be 4,200 mg/kg bw [Denine and Palanker, 1973]. For 4-*tert*-butylcyclohexyl acetate, the rat oral LD50 values were reported to range from greater than 500 to 5,000 mg/kg bw [Zeller and Hofmann, 1970; Moreno, 1976; Opdyke, 1976]. Similar values have been reported for 2-isopropyl-5-methylcyclohexanol (940 - 4,384 mg/kg bw) [Jenner *et al.*, 1964; Food and Drug Administration (FDA), 1975].

Dermal LD50s in rabbits were reported to be greater than 5,000 mg/kg bw for both 4-*tert*-butylcyclohexanol and 4-*tert*-butylcyclohexyl acetate [Denine and Palanker, 1973; Opdyke, 1976].

The mouse intraperitoneal LD50 for 4-*tert*-butylcyclohexanol was 50-100 mg/kg bw [Doull *et al.*, 1962] and for 4-*tert*-butylcyclohexyl acetate was 400 ml/kg bw [Zeller and Hofmann, 1970].

Given the current database of information, it will not be necessary to perform additional acute toxicity tests.

3.4.2 *In vitro* and *In vivo* Genotoxicity

Both chemicals in this category have been tested in *in vitro* bacterial and mammalian studies and have shown no mutagenic or genotoxic potential. Similar results have been reported for the isomeric cyclohexanol derivative, 2-isopropyl-5-methylcyclohexanol, for which a more extensive database was available. Although no *in vivo* genotoxicity data exist for 4-*tert*-butylcyclohexanol and 4-*tert*-butylcyclohexyl acetate, studies are available for 2-isopropyl-5-methylcyclohexanol and the results confirm the findings of the *in vitro* studies that the alkyl-substituted cyclohexanol derivatives exhibit a low genotoxic potential.

3.4.2.1 In vitro Genotoxicity

4-*tert*-Butylcyclohexanol and 4-*tert*-butylcyclohexyl acetate have shown no mutagenic potential when tested at concentrations up to 5,000 micrograms/plate in the Ames assay using *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, TA1538 with or without metabolic activation [Degussa AG, 1989, 1988a]. 2-Isopropyl-5-methylcyclohexanol was not mutagenic when tested in *Salmonella typhimurium* strains TA92, TA1535, TA100, TA1537, TA94, or TA98 with metabolic activation at concentrations up to 500 micrograms/plate [Ishidate *et al*., 1984] and when tested in *Salmonella typhimurium* strains TA100, TA2637 or TA98 with or without metabolic activation at concentrations up to 200 micrograms/plate [Nohmi *et al*., 1985].

The possible clastogenicity of 4-*tert*-butylcyclohexanol was studied in Chinese hamster V79 cells in the presence and absence of S9 [Degussa AG, 1997]. No biologically significant increases in chromosomal aberrations were reported and 4-*tert*-butylcyclohexanol was considered by the authors to be non-clastogenic in this experiment.

2-Isopropyl-5-methylcyclohexanol did not induce an increase in the incidence of chromosomal aberrations or an increased frequency of sister chromatid exchanges (SCEs) in human lymphocytes at concentrations up to 10 mM, with or without metabolic activation [Murthy *et al*., 1991], and also did not induce chromosomal aberrations in Chinese hamster fibroblasts when tested at concentrations up to 200 micrograms/ml [Ishidate *et al*., 1984].

As part of the National Toxicology Program (NTP), 2-isopropyl-5-methylcyclohexanol was not mutagenic when tested in *Salmonella typhimurium* strains TA100, TA1535, TA97, and TA98 with or without metabolic activation at concentrations up to 666 micrograms/plate [Zeiger *et al*., 1988], tested negative for chromosomal aberrations and SCEs in Chinese hamster ovary cells [Ivett *et al*., 1989], and did not increase mutation frequency in mouse lymphoma cells at concentrations up to 150 micrograms/ml or even at cytotoxic concentrations of 200 micrograms/ml [Myhr and Caspary, 1991].

2-Isopropyl-5-methylcyclohexanol was not clastogenic in human embryonic lung cultures when tested at concentrations up to 10 micrograms/ml *in vitro* [Food and Drug Administration (FDA), 1975].

3.4.2.2 In vivo Genotoxicity

Intraperitoneal injection on 3 consecutive days with up to 1,000 mg 2-isopropyl-5-methylcyclohexanol/kg bw did not induce micronuclei in mouse bone marrow [Shelby *et al*., 1993].

In a host-mediated assay, mice were gavaged with up to 3,000 mg/kg bw of 2-isopropyl-5-methylcyclohexanol in a single-dose study or up to 1,150 mg/kg bw/day of 2-isopropyl-5-methylcyclohexanol in a 5-day study [Food and Drug Administration (FDA), 1975]. After the last dose, mice were intraperitoneally injected with an indicator organism (*Salmonella typhimurium* strains G46 and TA1530, or *Saccharomyces cervisiae* D3). The peritoneal exudate was plated and incubated for assessment of mutation and recombinant frequencies. No significant increase in mutant and recombinant frequency was observed at any dose or exposure period in *Salmonella typhimurium* G46. In

Saccharomyces cervisiae D3, an elevation of recombinant frequency was reported in the 5-day exposure study, but not in the single-exposure study. At the highest dose tested in *Salmonella typhimurium* TA1530 in the single-dose study, a significant increase in mutant frequency was reported. This was not reported in the 5-day study. *In vitro* tests using the same organisms were all negative.

In a chromosomal aberration study, rats were gavaged with up to 3,000 mg/kg bw of 2-isopropyl-5-methylcyclohexanol as a single exposure or up to 1,150 mg/kg bw/day of 2-isopropyl-5-methylcyclohexanol for 5 days [Food and Drug Administration (FDA), 1975]. Analysis of bone marrow demonstrated that exposure to 2-isopropyl-5-methylcyclohexanol as a single dose or for a 5-day period did not induce chromosomal aberrations.

3.4.2.3 Conclusions

The *in vitro* studies on 4-*tert*-butylcyclohexanol and 4-*tert*-butylcyclohexyl acetate indicate that these substances exhibit no mutagenic or genotoxic potential. The isomeric cyclohexanol derivative, 2-isopropyl-5-methylcyclohexanol, with a larger safety database, also shows no genotoxic potential *in vitro* and these results are supported by findings in whole animals. Therefore, it is concluded that no additional genotoxicity studies are required for this chemical category.

3.4.3 Repeat Dose Toxicity

Repeat-dose toxicity studies are available for 4-*tert*-butylcyclohexanol and a mixture predominantly composed of isomeric alkyl-substituted cyclohexanol and cyclohexanone derivatives.

3.4.3.1 28-day Study for 4-tert-Butylcyclohexanol

Groups of rats were gavaged with 0, 50, 150, or 300 mg/kg bw/day of 4-*tert*-butylcyclohexanol for 28 days with a 14-day post-observation period [Degussa AG, 1999]. Clinical signs, body weight, food and water consumption were monitored during the study and after 28 days, behavioral tests were conducted and rats were killed and necropsied. Two high-dose rats died due to gavage error and were replaced. Clinical signs at the 2 highest doses included convulsions, squatting position, straub tail and vocalization. The signs disappeared within a few hours to 1 day. No clinical abnormalities were observed in the controls and rats in the 14-day observation period. After 2, 3 and 4 weeks of treatment, behavioral observations in individual rats (predominantly from the high-dose group) were made and included ataxia, fasciculations, padding movements, defense against touching, aggressiveness, hunchback/squatting position, reduced respiration, hyperactivity, straub tail, and slight convulsions. In the recovery period (week 5 and 6), no significant treatment-related clinical signs were observed in any treatment group. High-dose recovery group males showed a statistically significant increase of group mean values of landing-foot-splay and rearing and a decrease of group mean values of grip strength compared to recovery controls, but these differences were minor and did not show a consistent pattern in the individual animals. The effects were also not seen in the high-dose group males that were not allocated to the recovery group at the same examination time. Therefore the findings were considered of minor toxicological importance. No statistically significant increase in motor activity was observed in any dose group. A slight reduction in food consumption was seen in treated males, whereas, females showed an increase in food consumption when compared to controls. During the recovery period the food consumption of the treated animals was increased compared to controls. Water consumption was not different between treated and control groups. Alterations in clinical chemistry, urinalysis and hematology parameters were minor and within the normal range of the historical data. All differences observed were considered of minor toxicological importance. At the end of the treatment period high-dose male rats showed a statistically significant increase in relative adrenal weight when compared with controls. At the end of the recovery period, treated males exhibited a statistically significant increase in relative epididymis weight compared to the controls. However, there was no evidence of histopathology of this tissue. There were no other histopathological findings related to the reproductive organs of males (testes) or females (ovaries). Treatment-related histopathological findings were restricted to an increased number of male animals of the high-dose group with eosinophilic hyaline droplets in the epithelial cell cytoplasm of the proximal tubules (5 treated compared to 1 control). The authors proposed that the effect may be indicative of the *alpha*-2 micro-globulin nephropathy syndrome that is a rat-specific effect (see data and information included in study in section 3.4.3.2). Based on clinical signs, a no observable adverse effect level (NOAEL) of 50 mg/kg bw/day and a lowest observable adverse effect level (LOAEL) of 150 mg/kg bw/day were derived.

3.4.3.2 28-Day Study with Mixture of Alkyl-substituted Cyclohexanol and Related Ester Derivatives

A sample of an essential oil, predominantly containing a mixture of 2-isopropyl-5-methylcyclohexanol and 2-isopropyl-5-methylcyclohexanone isomers that accounts for greater than 85% of the mass of the oil, was used in the 28 day study [Serota, 1990] and reproductive/developmental screening [Hoberman, 1989] study cited below. Based on a gas chromatogram (FIS detector), the oil was determined to contain:

- 46.8% (1 *alpha*, 2 *beta*, 5 *alpha*)-2-isopropyl-5-methylcyclohexanol
- 3.97% (1 *alpha*, 2 *alpha*, 5 *alpha*)-2-isopropyl-5-methylcyclohexanol
- 0.86% (1 *beta*, 2 *beta*, 5 *alpha*)-2-isopropyl-5-methylcyclohexanol
- 21.81% (2 *beta*, 5 *alpha*)-2-isopropyl-5-methylcyclohexanone
- 3.07% (2 *beta*, 5 *beta*)-2-isopropyl-5-methylcyclohexanone
- 5.11% (1 *alpha*, 2 *beta*, 5 *alpha*)-2-isopropyl-5-methylcyclohexyl acetate
- 1.55% (1 *beta*, 2 *beta*, 5 *beta*)-2-isopropyl-5-methylcyclohexyl acetate

The other constituents accounting for approximately 10% of the oil included aliphatic terpene hydrocarbons (*e.g.*, *alpha*-pinene) and ethers (eucalyptol) (see Vollmuth, 1989 in Serota, 1990 reference).

The sample was administered by gavage in corn oil to groups of Sprague-Dawley rats at dose levels of 0, 100, 200, or 400 mg/kg bw/day for 29 or 30 days [Serota, 1990]. Clinical signs, body weights and food consumption were monitored. At necropsy, organ weights (brain, spleen, liver, heart, kidneys, testes with epididymides, adrenals, ovaries, and pituitary) were measured, and tissues (26) were preserved in 10% formalin. All tissues from the control and high-dose groups and tissues from the heart, liver, kidneys, and gross lesions from the low- and mid-dose group were embedded in paraffin, stained with hematoxylin and eosin, and examined microscopically. All animals survived to study termination with high-dose males showing increased incidence of urine staining during clinical observations. Except for a non-statistically significant decrease in mean body weight in high-dose males, there were no statistically significant differences in body weight or food consumption between treated and control groups. A significant decrease in serum glucose levels was reported in the mid- and high-dose males that the authors, in part, attribute to change in nutritional status as revealed by decreased body weights in the high-dose group. A treatment-related increase in alkaline phosphatase also was reported in high-dose males. Measurement of body weight, food consumption, hematology and clinical chemistry parameters revealed no significant changes between test and control female rats. There were statistically significant increases in relative kidney weights in high-dose males. Histopathological findings revealed renal tubule protein droplets in all groups of treated male rats. The authors considered these findings related to the lysosomal handling of *alpha*-2 micro-globulin, a protein specific to the male Sprague-Dawley rat. Absolute and relative liver weights in high-dose females also were significantly increased but these changes were not confirmed by histopathological examination. There was no histopathology of tissues from reproductive organs of males (testes with epididymis) or female (ovaries). Based exclusively on the renal pathology reported in all dosed groups of male rats, the authors concluded that the NOAEL for the sample is less than 100 mg/kg bw/day in male rats and 400 mg/kg bw/day in female rats.

3.4.3.3 Interpretation of alpha-2 Micro-globulin Data

The mechanism of *alpha*-2-micro-globulin formation in the male rat has been the subject of intensive research for the last decade. Because this mechanism of action is widely applicable to a broad range of compounds using different modes of administration, no robust summaries have been prepared for the research results described below.

Since publication of the reports on the 28-day studies on 4-*tert*-butylcyclohexanol [Degussa AG, 1999] and an essential oil containing a mixture of 2-isopropyl-5-methylcyclohexanol and 2-isopropyl-5-methylcyclohexanone isomers 2-isopropyl-5-methylcyclohexanol [Serota, 1990], the mechanism of action associated with the formation of *alpha*-2 micro-globulin in male rats has been extensively studied. It has been clearly demonstrated that renal lesions, which were also observed in numerous NTP studies, resulted from the accumulation of aggregates of *alpha*-2 micro-globulin (a low molecular-weight protein synthesized in the liver) and test agents or their metabolites in the P2 segment of the renal proximal tubule. This phenomenon was initially observed in the male F344/N rat (Strasser *et al.*, 1988; Borghoff *et al.*, 1990) but has now been identified in other well-recognized strains of laboratory rats (Hildebrand *et al*., 1997; Saito *et al.*, 1996).

The gene that encodes *alpha*-2micro-globulin has been isolated and the sequence deduced (Untermann *et al*., 1981). These proteins are expressed in the liver under hormonal control (Roy and Neuhaus, 1967; Wang and Hodgetts, 1998). *alpha*-2 Micro-globulin belongs to the *alpha*-2 micro-globulin super family of proteins that are characterized by a unique hydrophobic binding pocket. The lesions do not develop in the female rat or in humans (Bucher *et al*., 1986). Subsequent investigations have shown that the *alpha*-2 micro-globulin nephropathy found in the male rat does not develop in mammals that do not express the hepatic form of *alpha*-2 micro-globulin (Swenberg *et al*., 1989; Dietrich and Swenberg, 1991), mice (Bucher *et al*., 1986; Lehman-McKeeman and Caudill, 1994) and dogs (Webb *et al*., 1990).

Transgenic mice that express rat *alpha*-2 micro-globulin were tested for their ability to form hyaline droplets and develop nephropathies similar to their adult male rat counterparts (Lehman-McKeeman and Caudill, 1994). This study involved male rats as positive control, transgenic C57BL/6J mice as experimental group and native C57BL/6 mice as negative controls. The animals at age 70-75 days were placed in metabolic cages and received 150 mg/kg bw/day of *d*-limonene in corn oil by gavage for three days. Limonene is a potent inducer of renal nephropathy in adult male rats (Environmental Protection Agency, 1991; National Toxicology Program, 1990). Twenty-four (24) hours after the last dose the animals were sacrificed and the kidneys analyzed for evidence of nephropathy. Hyaline droplet formation was evaluated on a subjective scale, size and intensity (0-4) multiplied by tubular loading (0-3) for an overall scale of 0-12 with 12 being the most severe. In the absence of *d*-limonene the control groups transgenic mice and rats showed a hyaline droplet score of 1+/-0 and 6+/-0.5, respectively. The test transgenic mice and rats showed a hyaline droplet score of 2.5+/-0.3 and 11+/-1.3, respectively upon dosing with *d*-limonene. The native mice developed no signs of hyaline droplet formation and tested negative for presence of *alpha*-2 micro-globulin in their urine. The authors assert that based on the data presented "*alpha*-2 micro-globulin is the only protein that is involved in the etiology of hyaline droplet nephropathy".

An increase in the kidney-type-*alpha*-2 micro-globulin was seen in male Sprague-Dawley rats when these animals were administered 200 mg/kg bw/day of isophorone by gavage for 7 days. The increases in the urinary kidney-type-*alpha*-2 micro-globulin are dose-dependent and parallel-elevated accumulation in the kidney cells (Saito *et al.*, 1996).

In another study, adult male Wistar rats were administered two groups of chemical compounds, including 138 mg/kg bw of isophorone, potassium bromate, 2-propanol and a series of benzene and anthracene derivatives, to study induction of accumulation of *alpha*-2 micro-globulin and structure-activity relationships. A monoclonal antibody against *alpha*-2 micro-globulin was employed in a competitive ELISA procedure to determine its concentration in urine or tissue samples without purification. Plasma concentrations of *alpha*-2 micro-globulin were not significantly increased by any of the test compounds at 1 mmol/kg bw. Kidney tissue concentrations were found to be 297-300% higher than that of controls. The hyaline droplet accumulating (HDA) potential was dependent on the test compound but there was no relationship between HDA activity and the structure or the pathway used to metabolize the test substance (Hildebrand *et al*., 1997).

The above studies depend exclusively on histopathologic evidence to detect *alpha*-2 micro-globulin nephropathy. An *in vitro* assay based on the prerequisite that a chemical or metabolite bind to *alpha*-2 micro-globulin has been developed. The assay predicts, in greater than 90% (22/24) of the substances tested, the ability to induce *alpha*-2 micro-globulin nephropathy (Lehman-McKeeman and Caudill, 1999). *d*-Limonene-1,2-epoxide is well characterized as an *alpha*-2 micro-globulin nephropathy inducer and has a steady state binding constant (K_d) of 5 x 10^{-7} M (Lehman-McKeeman *et al.*, 1989). Based on this, a competitive binding assay was developed with [^{14}C]-d-limonene-1,2-epoxide and male rat urinary protein concentrate. Homogenous *alpha*-2 micro-globulin was obtained from adult male rats (Lehmann-McKeeman and Caudill, 1992). The assay was run with three series of competitive inhibitors terpenes (5), decalin/decanes (10), and halobenzenes (8). Total male urinary protein was incubated for 1 hour with the test materials, ranging from 0.001 to 3000 microM, and 0.5 microM [^{14}C]-d-limonene-1,2-epoxide. The ability of the test materials to displace 50% of the radiolabelled limonene epoxide from the protein was evaluated and IC50 values were calculated. An IC50 value of less than or equal to 100 microM for the terpene and decalin/decanone series is considered predictive of *alpha*-2 micro-globulin droplet formation. Substances with an IC50 calculated at higher than 100 microM in the competitive binding assay were subjected to microsomal oxidation to generate metabolites that would bind to *alpha*-2 micro-globulin. Three of the halobenzenes 1,2-, 1,4-, and 1,3-dichlorobenzene tested positive for *alpha*-2 micro-globulin binding when incubated in the presence of rat liver microsomes. Parallel *in vivo* tests were performed in rats and hyaline droplet formation in the kidney was assessed to confirm the *in vitro* results. The authors concluded that the *in vitro* assay is greater than 90% predictive of *alpha*-2 micro-globulin nephropathy induction in male rats without being invasive or requiring additional animal testing (Lehman-McKeeman and Caudill, 1999).

To further investigate kidney tissue concentration of *alpha*-2 micro-globulin in the lysosomal portion, intact kidney lysosomes were isolated from untreated or 2,2,4-trimethylpentane (TMP)-treated rats and their ability to take up *alpha*-2 micro-globulin was compared. It was found that *alpha*-2 micro-globulin could be directly taken up in the presence of the heat shock cognate protein (*hsc*73). Hsc73 contributes to the normal degradation, lysis, of *alpha*-2 micro-globulin in rat kidney and liver. However, in the presence of a chemical (TMP) known to induce aggregation of *alpha*-2 micro-globulin, the activity of this pathway is increased. This may be due to an increase in the concentration of a receptor protein in the lysosomal membrane, which accelerates the uptake of the cytosolic protein, *alpha*-2 micro-globulin (Cuervo *et al.*, 1999).

While humans produce low molecular weight serum proteins, which are reabsorbed by the kidney, there is no evidence that *alpha*-2 micro-globulin is produced (Olson *et al.*, 1990). Urine collected from adult male rats and humans revealed no evidence that *alpha*-2 micro-globulin production occurs in humans (Olson *et al.*, 1990).

It is unknown whether any human serum proteins possess a binding site similar to that of *alpha*-2 micro-globulin. Although this is a possibility, it appears remote, since female rats, mice, and dogs do not show the renal changes noted in male rats exposed to isophorone. It should be noted that there is a class of human proteins referred to as the *alpha*-2 micro-globulin related proteins. They appear to have no functional relationship to the adult male rat urine proteins. The human protein has a higher molecular weight, 25 kDa and is a component of a neutrophil gelatinase complex (Kjeldsen *et al.*, 2000; Triebel *et al*., 1992). An extensive review of the current scientific literature and genome databases reveals no native protein or biological entity that acts as a nephropathic agent like mature male rat *alpha*-2 micro-globulin. The accumulated evidence indicates that it is the unique anatomical, physiological, and biochemical properties of the male rat kidney, especially the proximal convoluted tubule, that allows isophorone to interfere with renal processing of the strain-specific *alpha*-2 micro-globulin. Therefore, this process is not predictive of human carcinogenicity. In a comprehensive review of *alpha*-2 micro-globulin nephropathy and associated renal tubule tumors produced in the male rat exposed to isophorone and other simple chemical substances (*e.g*., limonene, decalin and methyl isobutyl ketone), it was concluded that the F344/N male rat is not an appropriate model for assessing human renal carcinogenic risk (Environmental Protection Agency, 1991). After careful review, it has been concluded that the mechanisms leading to the renal carcinogenic findings in the male rat are largely known and strongly indicate that the nephropathy associated with male rats have no significance for human risk assessment (Burdock *et al*., 1990).

Based on the results of these studies, it can be concluded that the renal pathology reported in male rats treated with 4-*tert*-butylcyclohexanol or the mixture containing greater than 85% of alkyl substituted cyclohexanol derivatives is unrelated to the human health assessment. Therefore, with the exception of the renal effects reported in male rats, the NOAEL for male or female rats given the mixture of 2-isopropyl-5-methylcyclohexanol is 400 mg/kg bw/day and the NOAEL for male and female rats given 4-*tert*-butylcyclohexanol is 50 mg/kg bw/day.

3.4.3.4 Chronic Studies

3.4.3.4.1 Mice

B6C3F1 mice were fed diets containing 0, 930, 1870, 3750, 7500, or 15,000 ppm *dl*-2-isopropyl-5-methylcyclohexanol (approximately 0, 140, 281, 563, 1125 or 2,250 mg/kg bw/day of *dl*-2-isopropyl-5-methylcyclohexanol, respectively) for 13 weeks [National Cancer Institute, 1979]. Necropsies were performed on all animals at the end of the study. Histopathological examination was performed on tissues from selected animals. Six mice (sex not specified) died during the study but the deaths could not be attributed to compound administration. Final mean body weights of the male mice and female mice were not statistically different from those of the controls except for the high-dose female group which showed statistically significant decreased body weights. A slight increase in the incidence of perivascular lymphoid hyperplasia and interstitial nephritis was reported in female mice given the two highest dose levels. No adverse effects were reported for male or female mice administered 140, 281, or 563 mg/kg bw/day of *dl*-2-isopropyl-5-methylcyclohexanol.

A carcinogenicity study was conducted in which groups of B6C3F1 mice of each sex were fed diets containing 0, 2,000 or 4,000 ppm *dl*-2-isopropyl-5-methylcyclohexanol (approximately 0, 300, or 600 mg/kg bw/day, respectively) for 103 weeks [National Cancer Institute, 1979]. Necropsies and histological examinations were performed on all animals at the termination of the study and on those found dead during the study. The mean body weights of the treated mice were slightly lower than those of controls. Survival of the treated male mice and low-dose female mice was similar to the vehicle control animals; however, survival of the high-dose group of female mice was significantly less than that of the control animals but was not accompanied by any evidence of toxicity. There was no evidence of neoplastic or nonneoplastic lesions of the male (penis, prepuce, preputial gland, prostate, or epididymis) or female (uterus, endometrium, or ovaries) reproductive system. An increase in the incidence of hepatocellular carcinomas was observed in high-dose male mice, but was not statistically different from that observed historically in control mice of that age and strain (Haseman *et al*., 1986, no robust summary provided). A low incidence of alveolar/bronchiolar adenomas of the lung was observed in treated females but was not statistically different from the incidence of this neoplasm in historical control groups. Under the conditions of this study, the authors concluded that *dl*-2-isopropyl-5-methylcyclohexanol was not carcinogenic and did not produce any organ-specific toxicity for either sex of B6C3F1 mice at dose levels up to 600 mg/kg bw/day.

3.4.3.4.2 Rats

Fischer 344 rats were fed diets containing 0, 930, 1870, 3750, 7500, or 15,000 ppm *dl*-2-isopropyl-5-methylcyclohexanol (approximately 0, 93, 187, 375, 750 or 1500 mg/kg bw/day of *dl*-2-isopropyl-5-methylcyclohexanol, respectively) for 13 weeks [National

Cancer Institute, 1979]. Necropsies were performed on all animals at the end of the study. Histopathological examination was performed on tissues from selected animals. Final mean body weights of the male and female rats at all dose levels were similar to those of the controls. A slight increase in the incidence of interstitial nephritis was observed in high-dose male rats. This effect may have been related to the presence of *alpha*-2 micro-globulin, but at time of the study (*i.e.*, 1979) the *alpha*-2 micro-globulin phenomenon in the male rat kidney had yet been characterized. No adverse effects were reported for male or female rats administered up to 750 mg/kg bw/day of *dl*-2-isopropyl-5-methylcyclohexanol.

Fischer 344 rats of each sex were fed diets containing 0, 3,750, or 7,500 ppm *dl*-2-isopropyl-5-methylcyclohexanol (approximately 0, 187, or 375 mg/kgbw/day of *dl*-2-isopropyl-5-methylcyclohexanol, respectively) for 103 weeks [National Cancer Institute, 1979]. Necropsies and histological examinations were performed on all animals at the termination of the study and on those found dead during the study. The mean body weights treated rats were slightly lower when compared to the controls. Microscopic examination of tissues of test animals failed to reveal any evidence of neoplastic or nonneoplastic lesions, including those of the male (*e.g.*, penis, scrotum, prostate, mammary gland, or epididymis) or female (uterus, vagina, mammary gland, endometrium, or ovaries) reproductive system. Survival of the treated rats was similar to the control animals. Chronic inflammation of the kidney observed in the dosed older males was not considered by the authors to be related to the administration of *dl*-2-isopropyl-5-methylcyclohexanol since the effect is commonly observed in aged male Fischer 344 rats. There was no increase in the incidence of neoplasms of dosed females compared to that of control animals. In treated females, fibroadenomas of the mammary glands occurred at a lower incidence than in the control group. Alveolar/bronchiolar adenomas or carcinomas were reported only for the female control rats. There was no report of *alpha*-2 micro-globulin-induced nephropathy of male rats. This is not unexpected given that this phenomenon was identified only in subsequent NTP sponsored bioassays. Under the conditions of this study, the authors concluded that *dl*-2-isopropyl-

5-methylcyclohexanol was neither carcinogenic nor toxic for either sex of Fischer 344 rats at dose levels of up to 375 mg/kg bw of *dl*-2-isopropyl-5-methylcyclohexanol.

The extensive database of repeat dose studies for 4-*tert*-butylcyclohexanol and the isomeric alkyl-substituted cyclohexanol derivative, 2-isopropyl-5-methylcyclohexanol indicate that these substances exhibit no carcinogenic potential and a very low order of subchronic and chronic toxicity. Therefore, it is not necessary to conduct additional studies on 4-*tert*-butylcyclohexanol or 4-*tert*-butylcyclohexyl acetate.

3.4.4 Reproductive Toxicity

Virgin Crl CD rats were administered oral dose levels of 0, 150, 750, or 1,500 mg/kg bw/day of the 2-isopropyl-5-methylcyclohexanol by gavage for 7 days prior to cohabitation, through cohabitation (maximum of 7 days), gestation, delivery, and a 4-day post-parturition period. The duration of the study was 39 days [Hoberman, 1989]. The composition of the test material was identical to that used in the previously reported 28-day study [Serota, 1990]. The study design included measurement of parameters for reproductive and developmental toxicity. Maternal indices monitored included twice-daily clinical observation, measurement of body weights, food consumption, duration of gestation, and fertility parameters (mating and fertility index, gestation index, and number of offspring per litter). Offspring indices monitored included daily observation, clinical signs, examination for gross external malformations, and measurement of mortality (number of stillborns), viability (pups dying on days 1-4), body weight and body weight gain.

At the two highest dose levels, maternal mortality was increased; significant decreases in maternal body weight and food consumption were reported. Clinical observations of the dams included decreased motor activity, ataxia, dysnea, rales, chromorrhinorrhea, un-groomed coat and thin appearance, and significant increases in pup mortality. Live litters were reported for 9/19, 8/10, 5/6, and 1/4 pregnant females in the control, 150, 750, and 1,500 mg/kg bw/day groups, respectively. Increases in the numbers of dams with stillborn pups, stillborn pups, and late resorptions *in utero* were reported only in the mid-dose group. At the highest dose, 2 rats had only resorptions *in utero* when found dead on gestation day 23 and one rat possessed only empty implantation sites *in utero* on day 25 of presumed gestation. Even at the highest dose level, there was no evidence of an effect of the test article on implantation, duration of gestation, pup sex ratio, or gross morphology of pups. Based on these results the authors concluded that the maternal NOAEL for reproductive effects was 150 mg/kg bw/day and the offspring NOAEL for developmental effects is greater than 150 mg/kg bw/day, but less than 750 mg/kg bw/day.

In a dominant lethal assay, males rats were gavaged with up to 3,000 mg/kg bw of 2-isopropyl-5-methylcyclohexanol as a single exposure or up to 1,150 mg/kg bw/day of 2-isopropyl-5-methylcyclohexanol for 5 days [Food and Drug Administration, 1975]. Male rats were mated with 2 female rats per week for 7-8 weeks following the last treatment. Fourteen days after mating, females were killed and the uteruses examined for early deaths, late fetal deaths, and total implantations. No effect on early deaths, late fetal deaths or total implantations was reported when 2-isopropyl-5-methylcyclohexanol was administered to male rats prior to mating.

Given the lack of significant reproductive effects in the reproductive/developmental screening study and the absence of any significant effects to the reproductive organs of animals in subchronic and chronic repeat-dose studies, it is concluded that alkyl-substituted cyclohexanol exhibits a very low order of reproductive toxicity.

3.4.5 Developmental Toxicity

Teratology studies in four animal species were performed under Food and Drug Administration contracts for the isomer of 4-*tert*-butylcyclohexanol, 2-isopropyl-5-methylcyclohexanol. Studies in mice [Morgareidge, 1973a], rats [Morgareidge, 1973b], and hamsters [Morgareidge, 1973c] were performed using the same study design. In each study, virgin adult females (CD-1 outbred mice, Wistar rats, or golden hamsters) were mated with untreated young adult males and observation of vaginal sperm plugs was considered day 0 of gestation. Beginning on day 6 and continuing daily through day 15 (mice and rats) or day 10 (for hamsters) of gestation, groups (22-23 for mice, 22-25 for rats and 19-23 for hamsters) of pregnant females were given 2-isopropyl-5-methylcyclohexanol by gavage in corn oil. Mice received 0, 1.85, 8.59, 39.9, or 185 mg/kg bw/day, rats received 2.18, 10.15, 47.05, or 218 mg/kg bw/day, and hamsters received 0.05, 21.15, 98.2, or 405 mg/kg bw/day. Negative control groups received corn oil by gavage daily while positive control groups received aspirin. On day 17(mice), 20 (rats), or 14 (hamsters), all dams were subjected to Caesarian section and the number of live litters, implantation sites, number of resorptions, live fetuses, dead fetuses, and body weight of live pups were recorded. Gestation index, mortality, implant sites per dam, percent of live and percent partial live resorptions, litter size and weights, sex and sex ratio of pups, and gross abnormalities to pups were reported. The urogenital tract of each dam was examined for anatomical abnormalities. One-third of fetuses of each litter underwent detailed visceral examination at 10X magnification. The remaining two-thirds were stained with alizarin red S dye/KOH and examined for skeletal defects. No effects on any of the above-described parameters were reported in any of the species tested and the authors concluded that there was no evidence of maternal or developmental toxicity at dose levels up to and including 185 (mice), 218 (rats), and 405 (hamsters) mg/kg bw/day of 2-isopropyl-5-methylcyclohexanol during gestation.

Virgin adult female rabbits were artificially inseminated and beginning on gestation day 6 and continuing daily through day 18, pregnant rabbits were given 0, 4.25, 19.75, 91.7, or 425 mg/kg bw of 2-isopropyl-5-methylcyclohexanol by gavage in corn oil [Morgareidge, 1973d]. A positive control group received 2.5 mg/kg bw/day of 6-aminonicotinamide. On gestation day 29 all dams were subjected to Caesarian section and the number of *corpora lutea*, implantation sites, resorption sites, live fetuses, dead fetuses, and body weight of live pups were recorded. Gestation index, mortality, litter size and weights, sex and sex ratio of pups, and gross abnormalities to pups were recorded. The urogenital tract of each dam was examined for anatomical abnormalities. All live fetuses were placed in an incubator for 24 hours and evaluated for survival. All surviving pups were sacrificed and subjected to detailed visceral examination at 10X magnification. All fetuses were cleared with KOH, stained with alizarin red S dye, and examined for skeletal defects. As reported for the 3 other species, there was no evidence of either maternal toxicity or developmental toxicity at dose levels up to and including 425 mg/kg bw/day of 2-isopropyl-5-methylcyclohexanol. Given the results of this multiple species study, alkyl-substituted cyclohexanol derivatives exhibit a low potential for developmental toxicity.

3.4.6 New Testing Required

No further testing is required.

3.5 TEST PLAN TABLE

Chemical	Physical-Chemical Properties				
	Melting Point	**Boiling Point**	**Vapor Pressure**	**Partition Coefficient**	**Water Solubility**
CAS NO. 98-52-2 4-*tert*-butylcyclohexanol	A, Calc	A, Calc	A, Calc	A, Calc	A, Calc
CAS NO. 32210-23-4 4-*tert*-butylcyclohexyl acetate	A, Calc	A, Calc	A, Calc	A, Calc	A, Calc

Chemical	Environmental Fate and Pathways			
	Photo-degradation	**Stability in Water**	**Biodegradation**	**Fugacity**
CAS NO. 98-52-2 4-*tert*-butylcyclohexanol	Calc	NA	A	Calc
CAS NO. 32210-23-4 4-*tert*-butylcyclohexyl acetate	Calc	Calc	A	Calc

Chemical	Ecotoxicity		
	Acute Toxicity to Fish	**Acute Toxicity to Aquatic Invertebrates**	**Acute Toxicity to Aquatic Plants**
CAS NO. 98-52-2 4-*tert*-butylcyclohexanol	A, Calc	A, Calc	A, Calc
CAS NO. 32210-23-4 4-*tert*-butylcyclohexyl acetate	A, Calc	A, Calc	A, Calc

Chemical	Human Health Data					
	Acute Toxicity	**Genetic Toxicity *In Vitro***	**Genetic Toxicity *In Vivo***	**Repeat Dose Toxicity**	**Repro-ductive Toxicity**	**Develop-mental Toxicity**
CAS NO. 98-52-2 4-*tert*-butylcyclohexanol	A	A	R	A	R	R
CAS NO. 32210-23-4 4-*tert*-butylcyclohexyl acetate	A	A	R	R	R	R

Legend	
Symbol	**Description**
R	Endpoint requirement fulfilled using category approach, SAR
Test	Endpoint requirements to be fulfilled with testing
Calc	Endpoint requirement fulfilled based on calculated data
A	Endpoint requirement fulfilled with adequate existing data
NR	Not required per the OECD SIDS guidance
NA	Not applicable due to physical/chemical properties
O	Other

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